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Detection of Malonaldehyde-thiobarbituric Acid (MA-TBA) Complex by High Performance Liquid Chromatography (HPLC) in a Model System

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Abstract

Various concentrations of malonaldehyde (MA) produced upon hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) were reacted with 2-thiobarbituric acid (TBA) and the contents of MA-TBA complex were measured both with spectrophotometer and high performance liquid chromatography (HPLC). As the concentrations of MA-TBA increased, their absorbances and the corresponding HPLC peak areas increased. The correlation coefficient between absorbances and HPLC peak areas of MA-TBA complex exceeded 0.99. The treatment of cetrimide, an ion pairing agent, provided a complete separation of MA-TBA peaks from the other compounds and butanol extraction of the complex increased its recovery by 29.4%. Measurement of the content of MA-TBA complex for monitoring the development of lipid oxidation was proven to be successful with the use of high performance liquid chromatography.

Key words: *model system, high performance liquid chromatography, MA-TBA complex, ion pairng agent,* butanol extraction

INTRODUCTION

Among various methods measuring the development of lipid oxidation, thiobarbituric acid (TBA) value or thiobarbituric acid reactive substances (TBARS) absorbances are the most widely used. These methods are simple, sensitive (1) and have high correlations with the sensory evaluation data (2,3). These measure the amount of a secondary lipid oxidation product, malonaldehyde (MA, malondialdehyde, MDA), which forms a complex with thiobarbituric acid in acidic heating conditions (2,4). Malonaldehyde, a primary reactant with TBA, produces a red chromogen that has an absorption maximum at 532 nm. The absorption was measured with spectrophotometer and reported usually as either TBARS absorbances or mg of MA per kg of sample (2-5).

Despite a wide use of the TBA method for the measurement of lipid oxidation, it was indicated that this method is not specific because a wide variety of compounds other than MA from both lipid and nonlipid sources could react with TBA (5,6). Furthermore, TBA values reported from different laboratories have wide variations because each laboratory employs slightly modified conditions for the measurement of MA (1). In order to overcome these shortcomings, more specific and reliable analyses of malonaldehyde were attempted using high performance liquid chromatography (7-13). These methods were used measuring lipid oxidation development mainly in biological tissues such as plasma, liver and other tissues (1). Considering the fact that the compositions of these biological tissues and foods are similar, the application of HPLC for measuring lipid oxidation in foods would be successful.

This study was conducted to develop a more specific, reliable, reproducible and possibly less time-consuming method for measuring the amount of malonaldehyde in a model system using high performance liquid chromatography.

MATERIALS AND METHODS

Materials

Reagents used for MA-TBA analysis, 1,1,3,3-tetraethoxypropane (TEP), 2-thiobarbituric acid (TBA) and antifoam were obtained from Sigma Chemical Co. (St. Louis, USA). All the reagents were dissolved in HPLC-grade distilled water. HPLC-grade methanol and butanol were purchased from Merck (Darmstadt, Germany) and the ion-pairing agent, cetrimide (cetyltrimethylammonium bromide), was obtained from Sigma Chemical Co. for HPLC-analysis.

Release of MA from TEP and formation of MA- TBA complex

1,1,3,3-tetraethoxypropane was chosen for a model system producing free malonaldehyde. To 0, 2, 4, 6, 8 and 10×10^{-8} moles of TEP prepared, TBA was added (final vol. 10 ml). This mixture was heated in a boiling water bath for 35 minutes, which produces a red chromogen, the MA-TBA complex. Contents of MA in standard solutions were analyzed with both spectrophotometer and HPLC. The increase in absorbances and peak heights as well as the increase in MA concentrations were monitored.

Spectrophotometric determination of TBARS

The absorbances of MA-TBA complex formed in a TEP model system were measured with a spectrophotometer (UVIKON 922, Kontron instrument) at 532 nm.

HPLC analysis of MA-TBA complex

Analysis of MA released from a model system was per-

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formed with the Young-Lin 930 HPLC. The system was equipped with an UV-VIS variable wavelength absorbance detector (Young-Lin M720) and the detection was made at 532 nm. The separation was carried out on a μBondapak C_{18} column (3.9 mm × 30 cm, 10 μm, Waters) with the mobile phase of methanol and distilled water mixture.

Mobile phase

Various proportions, (a) 45:55 (b) 75:25 (c) 95:5, of methanol and distilled water mixtures were compared for the resolution of MA-TBA complex. Because MA-TBA complex is quite polar (1) and the mobile phase (a) is the most polar among the three, MA-TBA was never retained but just passed through the column, and as a result, no corresponding peak appeared. Mobile phase (c) gave better separation of the complex than mobile phase (a), but mobile phase (b) had the most balanced polarity and the best resolving power to separate MA-TBA complex (data not shown). Methanol and distilled water at a 75:25 proportion was chosen for the best composition of solvent and used for the analysis thereafter. Flow rate was 1 ml/ min and the column temperature was maintained at 30°C. Both solvents were filtered and degassing was carried out with a degasser (DEGASYS DG 1310, UNIFLOWS, Tokyo, Japan).

Effect of addition of an ion pairing agent on the resolution of MA-TBA complex

An ion pairing agent, cetrimide, was used because malonaldehyde exists mainly in enolic form in the aqueous solution (14) and it needs to be neutralized with cetrimide for a better resolution on a reversed phase column (15). Three concentrations, 0.1, 0.5 and 1.0% cetrimide were added to the mobile phase (w/v) and the powers of resolution were compared. Over 0.5% cetrimide, the mobile phase became too viscous for the HPLC to operate normally, therefore 0.1% cetrimide was chosen and added to the mobile phase later on. Chromatograms obtained after treatment with and without cetrimide were compred.

Effect of butanol extraction on the recovery of MA-TBA complex

It was reported that butanol extraction of MA-TBA is necessary to increase the recovery of the complex and to protect the column (7). The pH of the MA-TBA solution was adjusted to less than 0.75 with the addition of 6 N HCl in order to follow the procedure known to maximize the recovery of the complex (7). One ml of butanol was added to 5 ml of MA-TBA solution and the complex was extracted. After the butanol was evaporated with nitrogen gas at a temperature of 35°C, dried chromogen was resolubilized in 0.5 ml distilled water and 20 μl was injected into the HPLC.

UV-VIS DA (diode array) spectrophotometric analysis of HPLC eluant of MA-TBA

The HPLC eluant fractions representing MA-TBA complex were collected and the spectra of all wavelength were measured with UV-VIS DA spectrophotometer (HP 8453, Waldbronn, Germany). The above spectra were compared with those of the chromogens in the MA-TBA standard solutions manufactured with the hydrolysis of TEP in order to find out the homogeneity of the chromogen and the HPLC eluant for MA-TBA.

RESULTS AND DISCUSSION

Concentrations of MA vs spectrophotometric absorbances

Various concentrations of MA were prepared from TEP and the absorbances at 532 nm were measured. During heating, 1 mol of MA is released from each mol of TEP (16,17). Concentrations of malonaldehyde produced from TEP had a linear relationship with the absorbances as shown in Fig. 1.

HPLC separation of MA-TBA complex without treatment of cetrimide

Chromatograms of MA produced from TEP without treatment of cetrimide are shown in Fig. 2. Several components coeluted and an incomplete separation of MA-TBA was observed as shown in the figure. However, the peak area increased with the increase in concentrations of malonaldehyde (data not shown). Butanol extraction of the MA-TBA complex, which was reported to increase the recovery of the compex (7), did so to a certain degree (to be discussed later) but the separation was still not complete. For MA-TBA complex extracted with butanol, the increase in the peak areas of the complex in proportion to the concentrations was also observed.

HPLC separation of MA-TBA complex with the treatment of cetrimide

An ion pairing agent, cetrimide, was added to the mobile phase and the separation of the complex was examined. Ion pairing agents are used to improve the separation of ions which are poorly chromatographed in a reversed phase column (15). Malonaldehyde exists mainly in the anionic form in the solution (14) but after ion pair formation, it is separated as

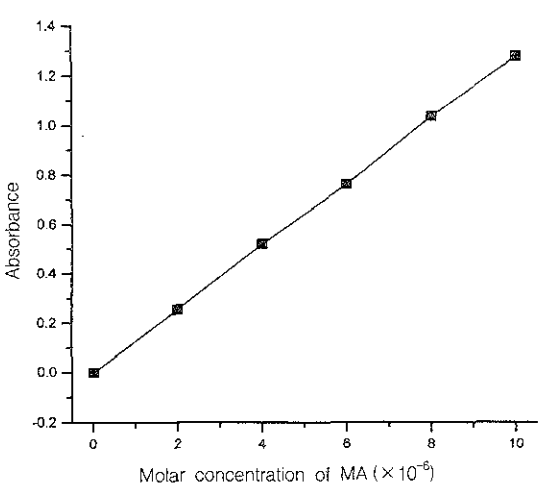

Fig. 1. Standard curve for MA (hydrolysis product of TEP)-TBA complex.

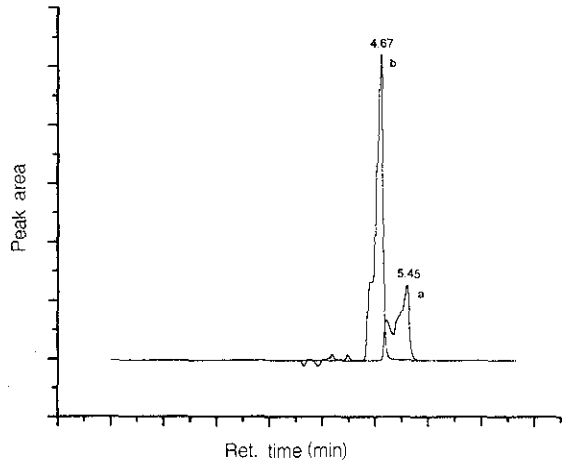

Fig. 2. HPLC elution profile of MA-TBA peaks w/o the treatment of cetrimide. a: not extracted w/butanol, b: extracted w/butanol.

neutral and it comes to have greater resolving power (Fig. 3). The retention time of the complex was delayed slightly (4~5 → 8~9 min), since the complex becomes neutral and polarity decreases and as a result, the complex remains in the column for a longer duration. As shown in Fig. 3, when the complex eluted, it had no interferences from the other compounds and the shapes of the peaks were typical well-separated single compound patterns. The recovery of the MA-TBA complex after butanol extraction also increased by a similar extent as mentioned in Fig. 2 (to be discussed later). The treatment of cetrimde for a complete separation of MA-TBA is an essential step and butanol extraction of the complex for increased recovery is stongly recommended.

Peak areas vs concentrations of MA-TBA standard solutions

As the concentrations of MA-TBA increased, peak areas of the complex increased proportionately (Fig. 4). Butanol-extracted MA-TBA also shows a similar increasing pattern with the non-extracted ones (data not shown). The retention time of the complex is between 8.0 to 9.0 minutes and the peaks eluted from the column with the help of cetrimide shows a well-separated pattern. When the peak areas of MA-TBA

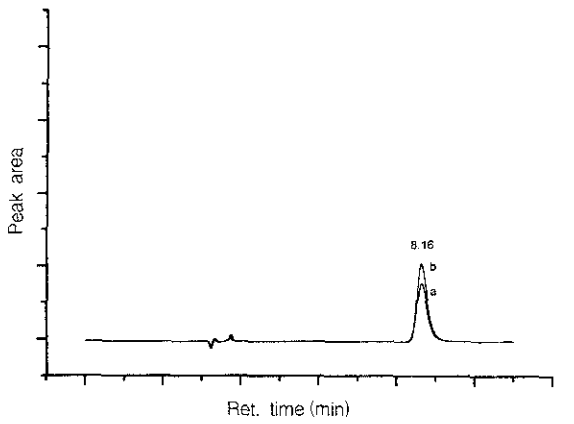

Fig. 3. HPLC elution profile of MA-TBA peaks w/ the treatment of cetrimide. a: not extracted w/butanol, b: extracted w/butanol.

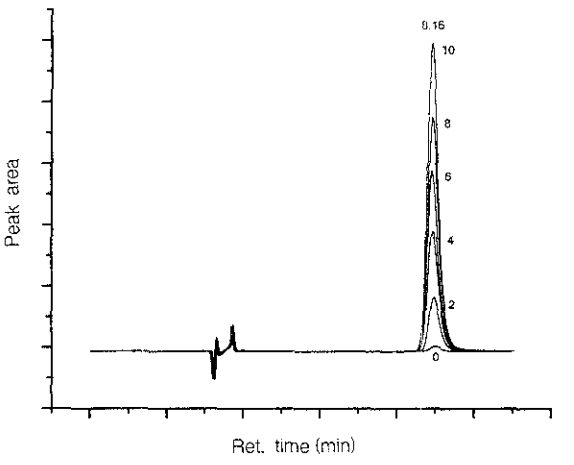

Fig. 4. HPLC chromatograms of various concentrations of MA-TBA complex. 0, 2, 4, 6, 8, 10 ($\times 10^{-6}$ molar concentration of MA-TBA, inj. vol. 20 μl).

were plotted against their concentrations, there were linear relationships between the two parameters (Fig. 5). The correlation coefficients between HPLC peak areas and the TBARS absorbances were 0.9981 for non butanol-extracted and 0.9954 for butanol-extracted MA-TBA complex. The treatment of butanol extraction increased the recovery of the complex by an average of 29.4% (Fig. 5). Summing up the observations, this HPLC method of MA-TBA separation with cetrimide treatment plus butanol extraction was successfully performed and can be directly utilized for analyzing MA-TBA in food systems.

UV-VIS DA spectra of the MA-TBA eluants

The eluant portions representing the MA-TBA peak was collected in repetition after several HPLC runs and the absorption spectra of the eluants were scanned with the UV-VIS DA spectrophotometer. The spectra for the color produced in MA-TBA standard solution itself were also plotted for a comparative purpose. As shown in Fig. 6, both materials show absorption maxima at 532 nm, which confirms that the HPLC

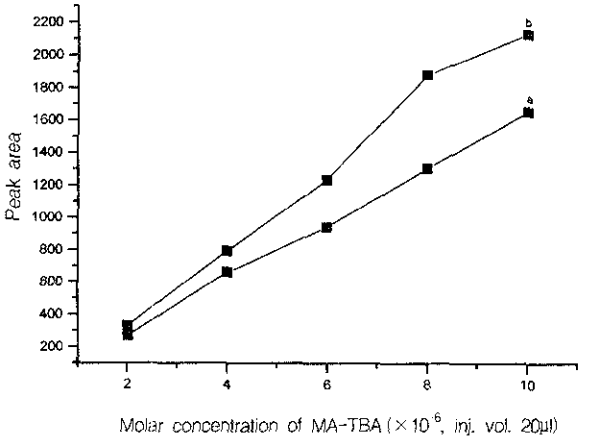

Fig. 5. Relationships between concentration of MA-TBA and their corresponding peak areas w/ (b) or w/o (a) the extraction of butanol (cetrimide treated). a: not extracted with butanol, b: extracted w/butanol.

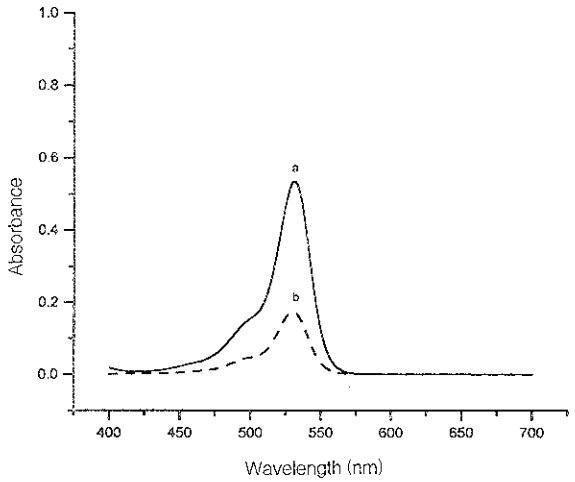

Fig. 6. UV-VIS DA spectra of (a) the colors formed between MA and TBA and those of the (b) HPLC eluant of MA-TBA fraction.

eluant for MA-TBA and the chromogen in the standard solution are the same compounds.

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